Plasmid/Strain	Properties	Reference
	Em^R	
pMutClpP	pMutin2 derivative; carrying the 5' part of the *B. subtilis clpP* gene; 8.9 kb; Ap^R; Em^R	This work
Strains		
E. coli		
TOP10	*F*⁻ mcr*A* Δ(mrr-hsd*RMS*-mcr*BC*) Φ*80*lacZΔ*M15* Δlac*X74* rec*A1* deo*R* *araD139* Δ(ara-leu)*7697* gal*U* gal*K* rps*L* (*Str*^{R}) end*A1* nup*G*	Invitrogen
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F¢*proAB lacI*^{q}Z*DM15* Tn*10* (Tet^r)]	Stratagene
B. subtilis		
168	*trpC2*	Kunst et al. 1997. The complete genome sequence of the Gram-positive bacterium *Bacillus subtilis*. Nature **390**:249-256.
168 Δ*ssrA*	*trpC2, ssrA* ; Sp^R	This work
168 I*ssrA*^{DD}	*trpC2*, I*ssrA*^{DD}; Tc^R; integration of pSsrADDTc in *ssrA::spec* in 168 Δ*ssrA*	This work
WB600	*trpC, nprE, aprE, epr, bpf, mpr, nprB*	Wu et al. 1991. Engineering a *Bacillus subtilis* expression-secretion system with a strain deficient in six extracellular proteases. J. Bacteriol. **173**:4952-4958.
BSE-23	*ctpA*; Sp^R	E. Lee, unpublished
WB600 Δ*ctpA*	*trpC, nprE, aprE, epr, bpf, mpr, nprB, ctpA*; Sp^R	This work
WB600 Δ*yvjB*	*trpC, nprE, aprE, epr, bpf, mpr, nprB yvjB*; Tc^R	This work
WB600 I*clpP*	*trpC, nprE, aprE, epr, bpf, mpr, nprB,* P*spac-clpP*; *clpP-lacZ* ; Em^R	This work
WB600 Δ*ssrA*	*trpC, nprE, aprE, epr, bpf, mpr, nprB, ssrA*; Sp^R	This work
WB600 I*ssrA*^{DD}	trpC, nprE, aprE, epr, bpf, mpr, nprB, I*ssrA*^{DD}; *Tc*^{R}	This work

Example 2

IL-3 Expression

When fused to the signal peptide of *B. licheniformis* α-amylase, human interleukin-3 can be secreted by *B. subtilis* (Van Leen et al. (1991), Biotechnology, 9: 47-52). Plasmid pLATIL3 contains the h-IL3 gene fused to the coding region of the *B. licheniformis* α-amylase (AmyL) signal peptide; in this plasmid expression of the hybrid AmyL-hIL3 gene is controlled by the *B. licheniformis* α-amylase promoter. During secretion, the AmyL signal peptide is removed from the AmyL-hIL3 precursor by signal peptidases, and mature hIL3 is released into the medium.

Expression of the human IL-3 gene lacking an in-frame stop codon in wild-type B. subtilis and in an ssrA mutant. Mutant 168 Δ*ssrA* was created, in which the *ssrA* gene is disrupted by insertion of a spectinomycin resistance cassette. The mutation was checked by PCR, and the absence of SsrA RNA in the mutant was confirmed by Northern blot analysis (Fig. 1A). Growth of 168 Δ*ssrA* was somewhat reduced compared to the wild-type strain (Fig. 1B), as reported recently by Muto *et al.* (2000. Requirement of transfer-messenger RNA for the growth of *Bacillus subtilis* under stresses. Genes Cells **5:**627-635). They also observed that growth rates of cells without SsrA decreased with elevating temperatures (> 45 °C). In addition, our results show that growth is more affected at low temperatures (< 25°C) then at temperatures between 30-45 °C (Fig. 2C), indicating a mild cold-sensitivity of growth in mutant 168 Δ*ssrA*.

Plasmid pLATIL3, a derivative of pGB/IL-322, contains an expression cassette for the production of human interleukin-3 (hIL-3) by Bacilli (Van Leen et al. 1991). In this construct, the *B. licheniformis* α-amylase (AmyL) signal peptide is used to direct secretion of mature hIL-3. As a model for SsrA-mediated peptide tagging in *B. subtilis*, a variant of plasmid pLATIL3 was created in which a transcription terminator is inserted into the *AmyL-hIL3*

gene, just in front of its stop codon. Transformation of this plasmid (pLATIL3TERM) into *B. subtilis* will result in *AmyL-hIL3* transcripts lacking in-frame stop codons. According to the tmRNA model for SsrA mediated tagging of proteins (Keiler et al. 1996), translation of these transcript will result in ribosome stalling, and subsequently recruitment of SsrA, peptide tagging, and finally degradation of the tagged hIL-3 molecules by specific proteases. To test this model in *Bacillus*, the extracellular proteins produced in cultures of *B. subtilis* 168 (pLATIL3TERM), 168 Δ*ssrA* (pLATIL3TERM), and the control strain 168 (pLATIL3), were analyzed by Western blotting (Fig. 2). Human IL-3 accumulated in the medium of strain 168 Δ*ssrA* (pLATIL3TERM), but could not be detected in the medium of *B. subtilis* 168 (pLATIL3TERM) containing functional SsrA. These data indicate that *B. subtilis* SsrA has a role in a process in which proteins translated from mRNAs lacking an in-frame stop codon are degraded. In contrast, in cells without SsrA the hIL-3 molecules are released from stalled ribosomes by an SsrA-independent mechanism (see below). These molecules do not receive a peptide-tag and, therefore are not rapidly degraded by B. subtilis.

RNA isolation and Northern blotting. RNA was isolated with the TRIzol method according to the protocol provided by the manufacturer (Life technologies), but with one modification: cells were incubated for 10 min at 37 °C with lysozyme (2 mg/ml) prior to lysis in TRIzol solution. Northern blotting was performed after electrophoresis of RNA through gels containing formaldehyde (Sambrook et al. 1989). To this purpose, Hybond-N+ nylon membrane from Amersham Pharmacia Biotech was used. The SsrA-specific probe was amplified by PCR with the primers SsrAFRWDP (5' ACG TTA CGG ATT CGA CAG GGA TGG 3') (SEQ ID NO:___) and SsrAREVP (5' GAG TCG AAC CCA CGT CCA GAA A 3') (SEQ ID NO:___). Labeling of the probe, hybridization and detection was performed with the ECL direct nucleic acid labeling and detection system from Amersham Pharmacia Biotech according to the manufacturer's instructions.